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Cobalamin speciation using reversed-phase micro-high-performance liquid chromatography interfaced to inductively coupled plasma mass spectrometry

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Abstract

Micro-high-performance liquid chromatography interfaced to inductively coupled plasma mass spectrometry was optimized for the determination and separation of a mixture of cobalt containing species. Four cobalamin species (cyanocobalamin, hydroxocobalamin, methylcobalamin, and 5′-deoxyadenosylcobalamin) representing the various forms of vitamin B12 as well as the harmful corrinoid analogue cobinamide dicyanide were separated using reversed-phase microcapillary chromatography with columns containing C18 packing material with a 2-μm particle size. Selection of organic solvents for the separation took into consideration compatibility with the inductively coupled plasma mass spectrometer being used for element specific detection. Optimized method conditions included use of a methanol gradient and make-up solution for the nebulizer. Some issues associated with dead volume were overcome by the extension of the gradient program. The total analysis time was 52 min. The column-to-column variability was evaluated and was found to be very reasonable (9% RSD on average), confirming that this method is rugged and that the technology should be easily transferred to other laboratories.

Keywords: Cobalamins; Vitamin B12; Reversed-phase; Micro-high-performance liquid chromatography; ICP-MS; Mira Mist CE

1. Introduction

Vitamin B12, isolated for the first time in 1946, is a generic name for a group of cobalt containing corrinoids known as cobalamins (Cbl) [1]. It is a symmetrical and complex molecule which resembles the structure of hemoglobin. In the hemoglobin molecule the central atom is Fe while for cobalamins it is Co. Depending upon the functional group (e.g. Cyano, CN-; hydroxo, OH-; methyl, Me-; adenosyl, Ado-) link to the β position of Co, different chemical species are formed. Naturally occurring cobalamins are only found in animal products such as meat, milk and dairy products. In meat the predominant forms are hydroxocobalamin (OH-Cbl) and 5′-deoxyadenosylcobalamin (Ado-Cbl) and in milk products hydroxocobalamin and methylcobalamin (Me-Cbl) are the prevalent ones. Other sources of cobalamins are food supplements and fortified foods which primarily contain the synthetic form cyanocobalamin [2]. Typical levels of cobalamins in food range from 3 to 250 ng/g and fortified cereals contain as much as 300 ng/g.

In the most recent daily reference intake (DRI) report from the National Academy of Sciences, the vitamin B12 recommended daily allowance is 2.4 μg/day [3] for adults. In that report, an assumption had to be made regarding the absorption of naturally occurring vitamin B12 due to the lack of data on dairy foods and most forms of red meat and fish regarding absorption of dietary sources. The report also identified the need to look at potential adverse effects with fortification of grain products, considering degradation products. This highlights the importance of being able to measure the various vitamin B12 species found in foods and dietary supplements to gain insights related to absorption and bioavailability.

Deficiency of vitamin B12 has been linked to a variety of health problems including hematological disorders and neuro-

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logical disorders. Vitamin B12 plays a role in many important functions acting as a coenzyme for normal DNA synthesis, promoting growth and cell development, promoting normal fat and carbohydrate metabolism as well as protein synthesis, and it plays a role in development and maintenance of the myelin sheath surrounding nerve cells. Populations at high risk of vitamin B12 deficiency are the elderly, vegetarians and people who have had gastric bypass surgery [3,4]. There is interest in knowing the exact form in both foods and biological samples to better understand the biosynthetic conversion of vitamin B12 to its coenzyme forms. Likewise, in the pharmaceutical industry and the food supplementation industry, it is important to have methodology which allows the individual cobalamins to be quantified since the stability of the individual cobalamin species is variable and breakdown products may occur, making some preparations less bioavailable to consumers. Again, based on these issues there is a lot of interest in developing speciation methods for the separation and quantification of individual cobalamins to evaluate and determine the forms and levels of the different species present in food samples and dietary supplements.

The "official" methods for the quantification of vitamin B12 are microbiological assays which are time-consuming to perform. In Methods of Analysis for Nutrition Labeling [5], three official methods are identified (AOAC Method 960.46, 952.20, and 986.23) which are suitable for the determination of vitamin B12 in a wide range of food matrices and vitamin preparations. In all instances, the cobalamins are converted to cyanocobalamin using KCN prior to microbiological analysis so individual cobalamin species cannot be measured.

A rapid and reliable approach is needed for the determination of important cobalamin species and a logical approach is the combination (hyphenation) of modern separation techniques with spectroscopic detection. Literature reports show that high-performance liquid chromatography is useful for these separations[6–12]. The commonly used systems of detection have been UV/Vis [6–9] and, to a lesser extent, inductively coupled plasma mass spectrometry [10–12]. Lambert et al. [6] reported on the use of capillary electrophoresis (CE) combined with UV/Vis detection and Baker et al. [13] combined CE with inductively coupled plasma mass spectrometry (ICP-MS) with good success. The long-term goal of this cobalamin project has been the evaluation of capillary-based microseparation techniques including CE, micro-high-performance liquid chromatography (μHPLC) and electrochromatograpy which is one of the separation modes of CE. The benefits of microseparation techniques include: low sample and stationary phase requirements and high separation efficiency [14]. When combining these microseparation techniques with ICP-MS possible benefits include: signal enhancement with the use of an organic make-up solution and increased flexibility with gradient programming using organic solvents with μHPLC-ICP-MS [15].

Modern analytical methods of preference for analysis of vitamin B12 species have been either conventional HPLC (4.0+ mm i.d.) or microbore HPLC (0.5–2.0 mm i.d) [16]. In particular, reversed-phase conditions using either C8 or C18 columns have been used with acetonitrile or methanol as the organic solvent components of the mobile phase [6–12]. When using ICP-MS as a detector, one must be aware of the effects of organic solvent which can lead to changes in analyte sensitivity as well as an increase in the number of carbon containing polyatomic ions, and the deposition of carbon on the cones [17–20]. Concerns related to the use of organic solvents with ICP-MS have limited their use in the development of hyphenated techniques.

Microbore chromatography has recently received attention for interfacing with ICP-MS because of the reduced flow rates it provides [10–12,17]. One might expect that capillary-based (< 100 μm i.d.) microseparations would be even more appropriate because only nanoliter volumes of organic solvents reach the plasma so detrimental effects are minimized. As previously reported [15], organic solvents can be effectively used with capillary-based μHPLC-ICP-MS to provide enhanced performance. This work demonstrated that the use of a solvent such as methanol can lead to signal enhancement with the ICP-MS. Because of the very low chromatographic flow rates (nl/min), transient effects related to changing solvent concentrations as a result of the gradient program are inconsequential due to the larger make-up solution flow rates (μl/min) used to satisfy the nebulizer.

From a separations point of view, acetonitrile would be the logical first choice for the reversed-phase chromatographic separation. From an ICP-MS detection point of view, however, methanol is better tolerated by the plasma [10]. In this study, both acetonitrile and methanol were evaluated and reversed-phase (C18) and normal phase (Cyano) separations were considered.

For many years, there were significant concerns related to the precision obtainable with capillary-based μHPLC techniques but significant advances have occurred in the last 5 years. High quality columns are now available from a large number of companies and capillary packing techniques have improved so column-to-column variability is not an issue and good precision may be obtained using μHPLC separation methods.

In this study, an optimized method was developed for the determination of coblamins using μHPLC-ICP-MS. The two major areas of consideration in developing an optimized method for cobalamins included sample injection reproducibility studies and selection of optimum chromatographic conditions (selection of organic solvents, packing material composition and particle size). The successful baseline separation and quantification of cobalamins is demonstrated.

2. Experimental

2.1. Chromatographic instrumentation

The chromatographic system used was a TriSep 2000GV CEC (Unimicro Technologies, Pleasanton, CA, USA). A

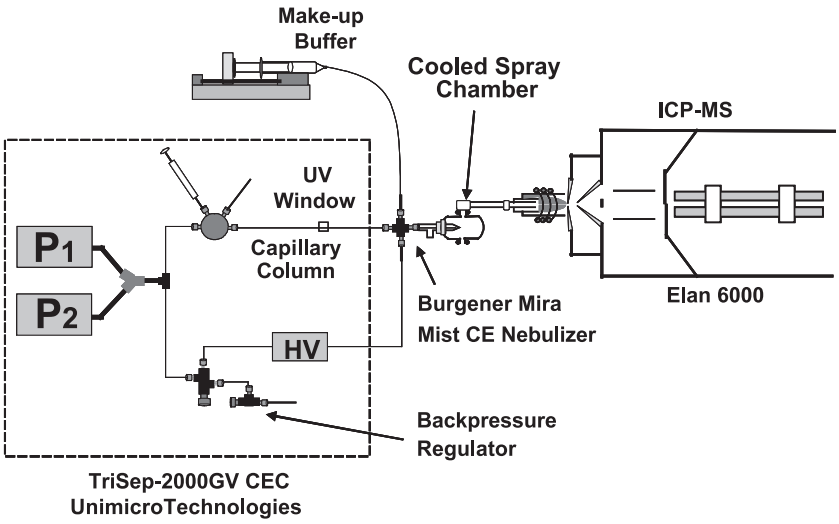

Fig. 1. System schematic for μHPLC-ICP-MS.

schematic of the system is shown in Fig. 1. The Unimicro Tri-Sep 2000GV consists of two pumps for gradient elution, a four-port valve injector and an on-line UV/Vis detector. In addition, it provides a high voltage power supply to allow pressurized capillary electrochrophoresis and capillary electrochromatography separations [21,22]. Packed capillary microseparation columns are directly connected to the four port injector valve (internal sample rotor volume of 20 nl). A continuous gradient flow enters the separation column through the valve injector and is maintained at a fixed pressure using a backpressure regulator. In this study, the instrument was modified to use an adjustable backpressure regulator to facilitate easily changing the column pressure for separation optimization. UV detection of cobalamins was achieved at 254 nm.

Chromatographic separations were achieved using reversed and normal phase conditions at room temperature. For reversed-phase conditions, a C18 column (Unimicro Technologies) with either 2 or 3 μm particle size packing material was used. For normal phase conditions, a Cyano column with 3 μm particle size was used. The packed capillary columns for this study were custom-made. Each was 365 μm o.d. and 75 μm i.d. The total length of the capillaries were 30 cm for UV detection and 37 cm for ICP-MS detection. Each column contained a 25-cm packed section sandwiched between two frits. The balance of the length of the capillary contained no packing material and provided either a UV window or the necessary connections to the ICP-MS.

2.2. ICP-MS instrumentation

The inductively coupled plasma mass spectrometer used in this work was an Elan 6000 (Perkin Elmer Sciex, Thornhill, Ontario, Canada). The ICP-MS was operated at 1.0 kW with a coolant gas flow rate of 15 l/min and an intermediate gas flow rate of 0.86 l/min. For cobalamin detection, the ^{59}Co isotope was monitored with a dwell time of 1 s. Data acquisition was initiated immediately after the sample was injected into the chromatographic system. The data were exported into Microcal Origin (Northampton, MA, USA) for processing. All quantitative data are based on peak area measurements.

Daily optimization of the ICP-MS system was achieved using both the PerkinElmer 10 ppb mutlielement tuning solution as well as a 10 ppb Co solution using the Mira Mist CE nebulizer.

2.3. Nebulizer and spray chamber

A Mira Mist CE nebulizer (Burgener Reserch, Mississauga, Ontario, Canada) coupled with a cooled cyclonic spray chamber (Glass Expansion, Camberwell, Australia) was used throughout [15]. The Burgener Mira Mist CE nebulizer is a low-flow, parallel-path nebulizer with specified sample flow rates from 1 to 2500 μl/min depending on the solution composition. This non-pneumatic nebulizer is designed particularly for interfacing CE with ICP-MS so it can easily accommodate any capillary-based technique combined with ICP-MS. It provides a four-way cross connected to the liquid inlet of the nebulizer. The capillary column which is inserted straight through the cross extends all the way to the tip of nebulizer. At this position the separation capillary column outlet is then recessed just enough ($\sim$ 1 mm) to allow the make-up solution to cover the capillary column and keep it wet. The capillary is held in place with a one piece finger tight-fitting. The make-up solution is introduced using a syringe pump (KD Scientific, New Hope, PA, USA) through Teflon® tubing attached to one of the cross arms. The fourth arm holds a platinum wire electrode needed for the electrical connection for CE or CEC separations.

Operating conditions were optimized [15] resulting in the use of a nebulizer gas flow rate of 1.0 l/min argon, a nebulizer pressure of 85 psi, and a make-up solution flow

15 μl/min. In addition, the jacketed Glass Expansion cyclonic spray chamber was used to provide cooling and the recirculating water temperature was set to 5 °C and maintained using a Model 911 recirculating chiller from PolyScience (Niles, IL, USA). A Miniplus peristaltic pump (Gilson, France) was used to drain the waste.

2.4. Reagents and standards

Analytical reagent grade chemicals were used throughout without additional purification. Deionized distilled water (18 MΩ) was used for all solution preparation and dilutions. Ammonium acetate was obtained from Fluka (Ronkondoma, NY, USA). Methanol, acetonitrile and sub-boiling distilled nitric acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Acetate buffers were prepared by dissolving 25 mM ammonium acetate in deionized, distilled water (or in 50% methanol or acetonitrile) and then adjusting to pH 4.0 with nitric acid. For gradient elution separations the following mobile phases were prepared: Mobile phase A was 25 mM ammonium acetate in water; Mobile phase B was 25 mM ammonium acetate in 50% acetontirile; and Mobile phase C was 25 mM ammonium acetate in 50% methanol. All solutions were filtered through a 0.45-μm Whatman filter (Clifton, NJ, USA) and de-gassed using an ultrasonic bath.

New μHPLC columns were conditioned using a 15-min gradient program starting with 50% methanol and ending with 25% (or in the case of acetonitrile starting with 50% and ending with 7.5%) which matched the concentration used for analytical separations. To maintain optimum column performance from day to day, a solution of 50% mobile phase B (25% methanol) or 15% mobile phase C (7.5% acetonitrile) was continuously pumped through the capillary at 1 μl/min overnight.

Cyanocobalamin (vitamin B12, CN-Cbl), hydroxocobalamin (vitamin B12a, OH-), methylcobalamin (methylcoenzyme B12, Me-), 5′-deoxyadenosylcobalamin (coenzyme B12, Ado-), and cobinamide dicyanide [$(CN)_2$-Cbn, Cob] were obtained as crystalline materials from Sigma (St. Louis, MO, USA) and were continuously stored in either the refrigerator or freezer as required to ensure stability. Stock solutions were prepared under low-light conditions by dissolving 10 mg of the substances in 10 ml of deionized, distilled water. Samples were stored in dark bottles at 2 °C. Working standard mixtures (200 ppm) were prepared before analysis by appropriate dilution of the stock standard solutions and stored in the refrigerator.

3. Results and discussion

3.1. Sample injection

The TriSep 2000GV CEC sample injector valve (with internal volume of 20 nl) was evaluated prior to separation optimization. Poor sample injection reproducibility is one of the reported weaknesses of capillary-based microseparation techniques. Yao et al. [22] highlighted the difficulty with fabrication of a small volume injector without introduction of a significant dead volume. The sample injector valve was characterized using six consecutive injections of a methylcobalamin standard at 3-min intervals into a C18 column operating under isocratic conditions with 50% acetonitrile. The RSD for peak areas was 3.8%. To simulate normal chromatographic separation conditions in which the analyte is retained on the column packing material, the experiment was repeated using a 45-min gradient and the RSD was 4.1%. The conclusion was that with RSDs consistently better than 4%, reliable sample injection reproducibility is not a problem.

3.2. Selection of chromatography conditions

3.2.1. Effect of organic solvent

Based on literature reports [6,7,9,11,12] acetonitrile was initially used for the separation of the five analytes of interest. Acetonitrile is commonly used in reversed-phase conditions because it provides good separation efficiencies (symmetrical and sharp peaks) and low column backpres-

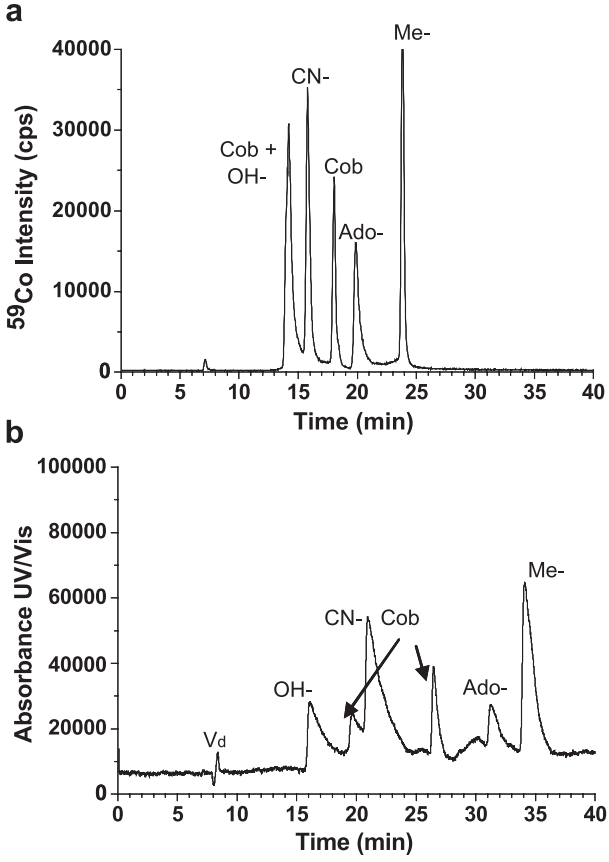

Fig. 2. Chromatograms for reversed-phase μHPLC (C18, 3 μm) showing the effect of organic solvent: (a) ICP-MS Detection, Acetonitrile, Gradient Elution Program = 40 min total: 15–45% B, 13 min; 45–100% B, 7 min; 100% B, 6 min; 100–50% B, 10 min; 15% B, 4 min. (b) UV/Vis detection; Methanol, Gradient Elution Program = 30 min total: 50% C 10 min; 50–80% C, 10 min; 80% C, 4 min; 80–50% C, 3 min; 50% C, 3 min.

sure due to its low viscosity. Fig. 2a shows the chromatogram of the separation of a mixture of four 200 ppm cobalamin standards (OH-Cbl, CN-Cbl, Ado-Cbl and CN-Cbl) and cobinamide dicyanide (Cob). In this particular study, results were obtained with a C18 capillary column with 3 μm particle size. The flow rate was 30 μl/min providing a column pressure of ~2500 psi. The ^{59}Co signal was monitored using the ICP-MS detector. Other separation conditions are shown in the figure legend. Peak identification was done by comparing retention times obtained from injection of individual standards and confirmed by spiking as necessary. The cobinamide dicyanide is present in two peaks. This is expected at pH 4, as previously reported by both Lambert [6] and Chassigne [10]. Chassigne and Lobinski [10] hypothesized that at a pH of ≤5, cobinamide dicyanide split into two isomeric forms in which the difference is the axial positioning of cyanide. They supported this conclusion using ESI-MS which produced identical spectra for both peaks.

As shown in Fig. 2a, one the isomeric forms of cobinamide dicyanide overlaps with hydroxocobalamin (OH-). Despite extensive attempts to optimize experimental conditions, considering pH, gradient programming and column pressure, the overlapping peaks could not be resolved. Although the literature indicates that a single peak for cobinamide dicyanide can be achieved at a pH of 6.8, no set of experimental conditions from this study provided reproducible separations. This may be the result of a pH effect as a result of working outside the optimum pH range of the buffer system, creating an unstable interaction between the cobalamin species and the stationary phase. Despite significant efforts to optimize conditions, no acceptable acetonitrile-based μHPLC separation could be achieved for the five cobalamin species of interest.

The usefulness of methanol as an organic solvent for the separation of cobalamin species has been demonstrated previously [8,10,11] using non-capillary-based separation techniques. For the sake of simplicity, while establishing optimum μHPLC separation conditions, several experiments were conducted using conventional UV/Vis detection. Fig. 2b shows a UV absorbance chromatogram (254 nm) of a separation of the mixture of four 200 ppm cobalamin standards (OH-Cbl, CN-Cbl, Ado-Cbl and CN-Cbl) and cobinamide dicyanide. The use of methanol caused a substantial degradation of the separation when compared with acetonitrile as evidenced by poor peak shape and symmetry for most of the analyte peaks. The elution order is similar as obtained using acetonitrile, but now the cobinamide dicyanide is split into two peaks, one of which overlaps with cyanocobalamin instead of hydroxocobalamin. The conclusion reached was that with careful optimization of separation conditions, it was likely that the cobinamide dicyanide and cyanocobalamin peaks could be resolved while retaining the resolution of the peaks from the other species. Because methanol is also tolerated better by the ICP-MS, it was selected as the solvent of choice for the balance of this study.

3.2.2. Effect of packing material

Normal phase separations using a cyano column were also evaluated with the hope that by changing the elution order of the cobinamide dicyanide and cobalamin species, that the co-elution problems encountered with the C18 column could be avoided. A representative UV/Vis (254 nm) chromatogram is shown in Fig. 3. The cyano column had the same dimensions as the C18 columns: an i.d. of 75, o.d. of 360 μm, a total packed section of 25 cm and the particle size was still 3 μm. Separation was performed using a gradient elution program as specified in the figure legend.

As expected the elution order was different from that obtained with reversed-phase conditions. Peak identification for the mixed standards chromatogram was confirmed by comparison with the chromatograms of the individual cobalt-contains species. Unfortunately, the goal of avoiding problems related to co-elution was not realized. As can be seen in Fig. 3, cobinamide dicyanide now co-elutes with both hydroxocobalamin and adenosylcobalamin. Attempts were made to further optimize the gradient to improve the separation without success. As a result, the balance of the study focused on reversed-phase (C18) separations.

3.2.3. Effect of packing material particle size

Since reversed-phase (C18) separations combined with methanol seemed most promising, a careful evaluation of parameters which might improve the separation was made. The only identifiable conditions which might help to resolve the overlap of the cobinamide dicyanide and cyanocobalamin peaks were the particle size of the packing material and the length of the packed column. It was expected that a reduced particle size could provide increased surface area for interaction improving the separation efficiency. Custom-packed C18 columns utilizing a particle size of 2 μm was used. The experimental conditions are similar to the ones previously used with 3 μm particle size packing material with the exception that the column pressure was 2700 psi. Fig. 4 shows a UV absorbance chromatogram of the

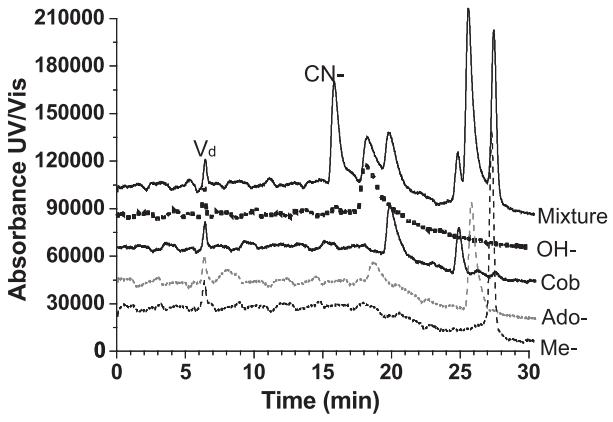

Fig. 3. Chromatograms for normal phase μHPLC using (Cyano column, 3 μm). Gradient Elution Program = 30 min total: 40% C, 10 min; 40–90% C, 10 min; 90% C, 4 min; 90–40 C, 3 min; 40% C, 3 min.

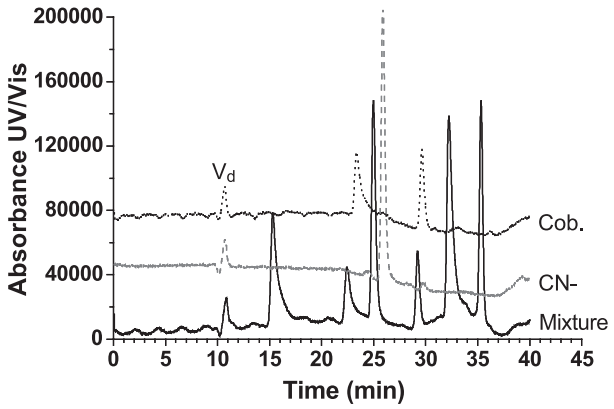

Fig. 4. Chromatograms for reversed-phase μHPLC (C18, 2 μm) showing the separation of a mixture of standards as well as individual injections of cobinamide dicyanide and cyanocobalamin. Gradient Elution Program = 50% C, 10 min; 50–90% C, 10 min; 90% C, 4 min; 90–50% C, 3 min; 50% C, 3 min.

successful baseline separation of the different cobalt containing analytes using the smaller (2 μm) diameter packing material. Fig. 4 also shows the chromatograms of individual injections of cyanocobalamin as well as cobinamide dicyanide. Calculated RSDs were better than 5% for both retention times and peak areas.

3.2.4. Effect of dead volume

It is a common practice that when interfacing conventional HPLC with ICP-MS, a transfer line is required to transport the effluents coming from the separation column to the sample introduction system of the ICP-MS. This transfer line should provide minimum dead volume in order to maintain the integrity of a particular separation [19]. In this study, the packed capillary column inserts directly into the nebulizer of the ICP-MS. As such, there is no need for a separate transfer line. An increase in the total column length from 30 to 37 cm was inevitable because of the distance between the μHPLC system and the ICP-MS.

Fig. 5 shows chromatograms for both UV/Vis and ICP-MS detection. Please note that the retention time for the hydroxocobalamin peak is approximately 5 min later for ICP-MS detection as a result of the 7 cm longer column length. In addition, the resolution is degraded. A careful evaluation of the system geometry and review of previous experiments indicated that the dead volume could not be significantly reduced because the microseparation system and the ICP-MS are as close together as possible.

An alternative approach to solving the problem of decreased resolution obtained with ICP-MS detection was to increase the analysis time by extending the gradient program. It was expected that this approach would provide acceptable resolution even with the present dead volume. The chromatogram of the successful separation of the cobalamin species and cobinamides obtained with ICP-MS detection is shown in Fig. 6. Optimum experimental conditions resulting in baseline separation, are summarized in the figure legend. These conditions resulted in an overall

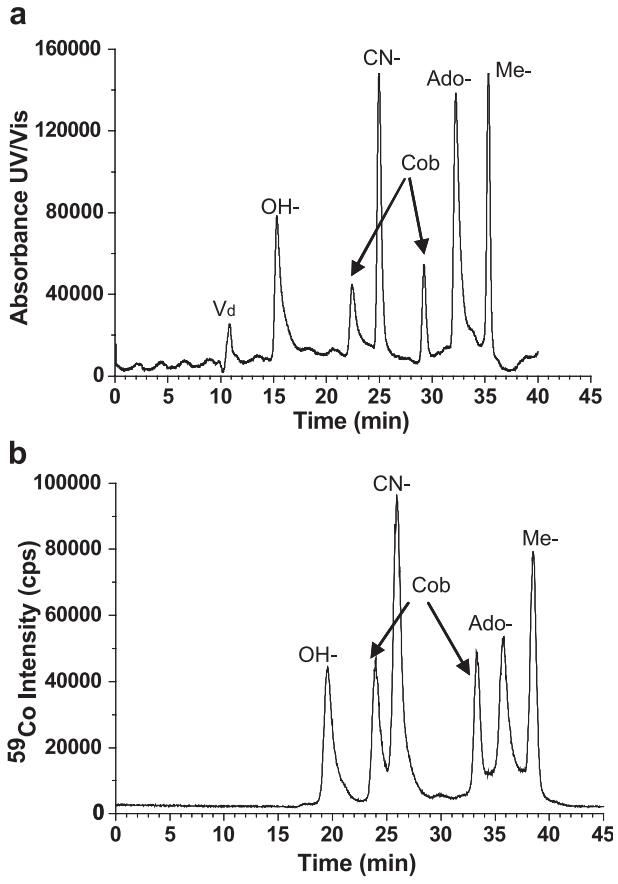

Fig. 5. Chromatograms for reversed-phase μHPLC (C18, 2 μm) considering dead volume issues. Gradient elution program same as Fig. 4: (a) UV/Vis detection; (b) ICP-MS detection.

increase of 11 min in analysis time. These experiments confirm that the poor resolution with ICP-MS detection is due to the extended capillary length and is not due to the nebulizer interface when using the Mira Mist CE nebulizer since the capillary column extends all the way to the tip of the nebulizer. Other nebulizer designs evaluated in the past

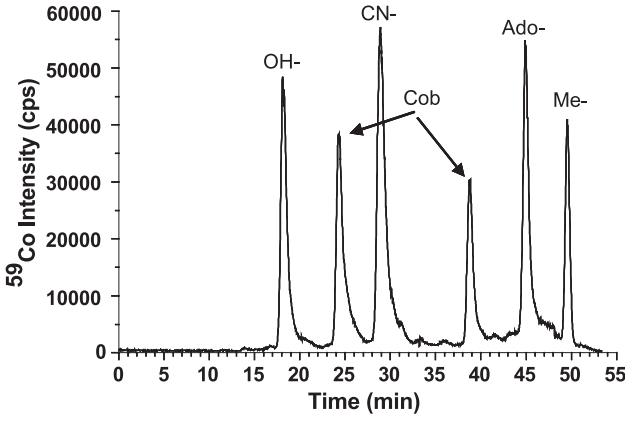

Fig. 6. Chromatogram for optimized reversed-phase μHPLC-ICP-MS (C18, 2 μm). Gradient Elution Program: 50% C, 15 min; 50–90% C, 18 min; 90% C, 3 min; 90–50% C, 2 min; 50% C, 3 min.

provided measurable contributions to the overall dead volume of the system.

Review of the chromatogram in Fig. 6 provides additional insights related to the system dead volume. Up to this point, discussions related to dead volume issues have focused on the extension of the capillary column to facilitate interfacing with the ICP-MS. Another important concern is the dead volume present before the inlet of the capillary column (between the pumps and the valve) as well as the dead volume within the valve. Looking at the gradient program, one would expect if there was no significant dead volume that all of the peaks would have eluted by the end of the gradient (36 min). It was demonstrated that the dead volume associated with the 7 cm extension of the capillary for interfacing to the ICP-MS leads to a 5-min delay so one would expect all peaks to have eluted by 41 min. The additional 10-min delay (elution completed at 51 min) observed can be attributed to the "front end" dead volume of the system. This is further confirmed by looking back at Figs. 4 and 5a where the dead volume peak (V_d) for UV/Vis detection is clearly identified as occurring at 10 min.

The physical limitation in the existing system contributing to the "front end" dead volume is due to the fact the capillary cannot be inserted all the way into the rotary injection valve [22]. Several options have been discussed with the manufacturer of the TriSep 2000GV CEC concerning reduction of the dead volume in the system for use with μHPLC determinations including the replacement of the four-port valve injector by a six-port valve and the re-plumbing of the microfluid system including a flow splitter. This is an important area of ongoing research.

One might expect that having no dead volume peak would be ideal. That is not the case. A dead volume peak is sometimes desirable because it will serve a useful purpose when analyzing real samples with complex matrices, avoiding interferences with the separation process due to impurities in the sample. Minimizing the dead volume to the greatest extent possible will provide two benefits. First, it will lead to improved separation efficiency as evidenced by symmetrical peak shapes. This will result in improved analytical precision for quantification. Next, it will reduce the overall analysis time.

Table 1
μHPLC ICP-MS cobalamin method column reproducibility study

		OH-	Cob #1	CN-	Cob #2	Ado-	Me-
Within-day precision study (14-Nov)							
Retention times		19.25	25.86	30.24	40.07	45.26	49.46
		19.15	25.67	29.24	39.72	44.79	49.23
		18.74	24.57	28.79	39.44	46.64	49.00
	Mean T_R	19.05	25.37	29.42	39.74	45.56	49.23
	SD	0.270	0.696	0.742	0.316	0.962	0.230
	RSD%	1.42	2.75	2.52	0.79	2.11	0.47
Peak areas		37,248	33,839	71,675	25,235	38,462	33,574
		39,118	34,783	74,344	25,014	39,146	31,192
		36,095	34,385	76,566	25,773	40,827	34,952
	Mean peak area	37,487	34,336	74,195	25,341	39,478	33,239
	SD	1526	474	2449	390	1217	1902
	RSD%	4.07	1.38	3.30	1.54	3.08	5.72
Between-column precision study							
Retention times							
14-Nov		19.05	25.37	29.42	39.74	45.56	49.23
10-Nov		21.06	25.96	28.33	40.33	46.70	51.79
6-Nov		18.11	24.44	29.17	38.86	46.19	49.72
	Mean T_R	19.40	25.26	28.97	39.64	46.15	50.25
	SD	1.505	0.766	0.572	0.740	0.569	1.359
	RSD%	7.75	3.03	1.98	1.87	1.23	2.70
Peak areas							
14-Nov		37,487	34,336	74,195	25,341	39,478	33,239
10-Nov		32,060	30,336	79,069	26,369	38,204	31,999
6-Nov		38,713	37,022	62,918	22,157	42,256	36,362
	Mean	36,087	33,898	72,061	24,622	39,979	33,867
	SD	3541	3364	8284	2196	2072	2248
	RSD%	9.81	9.93	11.50	8.92	5.18	6.64

3.2.5. Column variability

Numerous literature reports have mentioned that capillary-based microseparation systems have developed slowly due to problems related to reliable production of packed columns. Recent technological advances have led to uniform packing methods resulting in reproducible production of columns [23]. Since the goal of this research was the development of a rugged and reliable μHPLC method which would provide reproducible results using columns from different production lots, studies were performed to characterize the precision of the method associated with the columns. Table 1 contains data showing the within-day performance of a typical column. The precision data from three replicate analyses of a cobalamin standard mixture were calculated. The within-day relative standard deviation (RSD) for the retention times ranged from 0.5% to 2.8%. For peak areas the RSDs ranged from 1.4% to 5.7%. Similar within-day precisions (0.2–1.2% RSD for retention times and 2.0–4.6% RSD for peak areas) were obtained for two other columns studied. To evaluate the between-column precision, the mean values for three repeated injections obtained for three different columns, each characterized on a different day, were used. As one can see, the between column RSD of the retention times was 1.2–7.8%. The values were not significantly larger than those obtained for the within-day precision study with the exception of the hydroxocobalamin and methylcobalamin peaks. No explanation could be found for the measurable variation in the retention times seen for hydroxocobalamin (OH-). The between-column RSDs for the peak areas ranged from 5.2% to 11.5% RSD. Because the between-column variability study was done on different days, the day-to-day variability is also included. It is clear that the peak area RSDs are poorer than the retention time RSDs. The processing of these data required manual identification of the integration limits for the peaks which may have led to slightly inflated RSDs. Nevertheless, the average between column precision was 9% RSD which is quite acceptable and demonstrates that the columns are reproducibly manufactured as evidenced by the overall precision obtained using this μHPLC method. Future experiments will utilize recently acquired TotalChrom® (PerkinElmer Instruments, Shelton, CT, USA) commercial chromatography software which should further improve the precision. This study confirms that a rugged and reliable capillary μHPLC method can be developed for separation and quantification of cobalamin species.

4. Conclusions

Capillary high-performance liquid chromatography has been successfully applied to the baseline separation of a mixture of five different cobalt containing species. Baseline separation was achieved using reversed-phase conditions with a C18 packed capillary column with a 2-μm particle size and a gradient elution program. The result is a chromatographic separation with six peaks with two representing the cobinamide dicayanide content. The μHPLC-ICP-MS method is significantly better than a conventional UV/Vis method because of the simple chromatogram that results when using an element specific detector for the analysis of food samples and supplements which contain very complex matrices. The use of ICP-MS detection requires an increase in the length of the gradient program leading to an overall increase in analysis time. This work demonstrated that reproducible injections could be achieved with the 20 nl injection valve but that dead volume issues associated with the use of the TriSep 2000GV CEC for μHPLC-ICP-MS in its standard configuration requires further investigation and system optimization.

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